THE PRODUCTION OF TWIN EMBRYOS IN DENDRASTER BY MEANS OF MERCAPTOETHANOL (MONOTHIOETHYLENE GLYCOL)[2]

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The problem of individuation in the earliest embryonic development of certain animal groups resolves itself into questions concerning the interaction of blastomeres. Some transaction *between* the blastomeres determines that the first division will produce an individual composed of two cells rather than two individual embryos. Physical contiguity is a factor by definition, for, in those cases where the blastomeres are capable of producing complete embryos, such "twinning" can always be achieved by complete separation of the blastomeres. But complete physical separation is not necessary for functional isolation of the blastomeres; from studies of echinoid eggs we have a variety of experimental conditions under which twin embryos are produced from sister blastomeres in contact with each other (summarized by Schleip, 1929; Harvey, 1940). The experimental problem is to define the means—not necessarily a single one—whereby adjacent cells can mutually influence or restrict each other's behavior. The question is of interest in research on cell division as well as on developmental problems, and probably has much broader implications relative to the behavior of multicellular systems. In the case of echinoid eggs, it has received a good deal of attention, particularly in studies on cell division, and some of the ideas regarding the mechanisms are reviewed in a paper by Dan and Ono (1952).

Our chemical insights into the mechanisms of blastomere interaction are rather rudimentary, centering on the study of "extracellular coats" or "intercellular cements" which have, for good reasons, been characterized as calcium proteinates.

The present work is part of a series of studies in which mercaptoethanol (monothioethylene glycol) was employed as an agent which was expected to interfere with the association of protein molecules through thiol groups. The considerations underlying the study and the selection of this agent are discussed in another paper (Mazia, 1958). It was found, with the eggs of *Dendraster excentricus*, that treatment with mercaptoethanol at the proper time would produce twins in very high yields even though the blastomeres remained in contact within the fertilization

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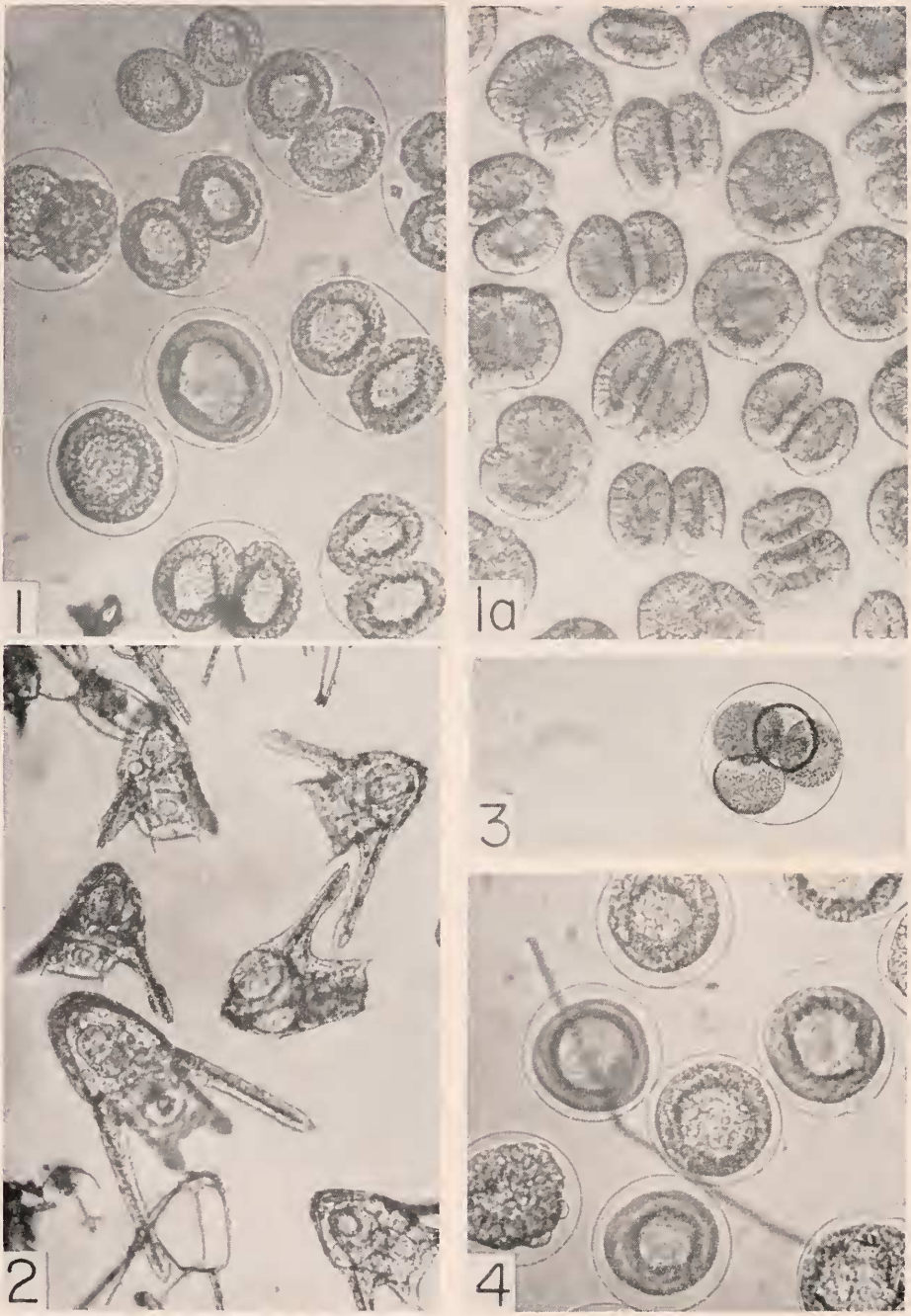

FIGURE 1. Twin blastulae. *Dendraster* eggs had been placed in 0.1 *M* mercaptoethanol in sea water at 41 minutes after insemination, and exposed for 28 minutes. Photographed alive at 7 hours 15 minutes after insemination. Twins are hatching in embryo at top of photograph.

membrane. The points of interest in the following discussion are not only the interpretation of the effect as one implicating thiol groups in blastomere interaction, but also the fact that processes determining the twinning or non-twinning may be restricted to a short period *during* the cleavage of the eggs.

Methods

The details of the methods used will be found in a previous paper (Mazia, 1958). The eggs of *Dendraster excentricus,* obtained in Mission Bay, San Diego, California, were used. At various times after fertilization, nine volumes of egg suspension were mixed with one volume of 1 *M* 2-mercaptoethanol (Eastman) in sea water. A common synonym for mercaptoethanol ($HSCH_2CH_2OH$) is monothioethylene glycol. After various times of exposure, the eggs were washed in sea water and their development was followed. When the fertilization membrane was to be removed, this was done by treatment with a solution of Worthington "Crude Protease" in sea water (0.1 mg. per ml.). In the case of the *Dendraster* egg, the protease may be introduced a few minutes after fertilization, and the dissolution of the fertilization membrane may be observed visually. The fact that the membrane of *Dendraster* eggs is susceptible to protease for some time after fertilization was called to my attention by Dr. William E. Berg.

Results

The over-all study of which this is a part concerned the blockage of mitosis by mercaptoethanol. The essential finding was that 0.1 *M* solutions would block division completely if applied at any time up to the time of metaphase, which takes place at about 35–40 minutes after fertilization, at 24° C. If the mercaptoethanol is applied at any time after this critical point, the cells divide without delay, and if left in the mercaptoethanol are blocked reversibly in the two-cell stage. In the course of observations on the reversibility of the block it was noted that a large proportion of those eggs which had cleaved while in the mercaptoethanol gave rise to twin blastulae when returned to sea water. Such a population containing the twin blastulae is shown in Figure 1. These blastulae gastrulate and develop into normal plutei (Fig. 2).

In order to obtain twinning, the mercaptoethanol must be applied during the period of furrowing. This is shown in Table I, where the yield of twins from eggs placed in mercaptoethanol at various times from metaphase to the completion of furrowing is given. At 35 minutes after fertilization, half of the eggs are blocked

Figure 1A. Another group of twin blastulae, fixed in 1 per cent formaldehyde in sea water. In this experiment, evidence of incomplete twinning is seen in some individuals.

Figure 2. Plutei produced by twinned embryos. The small plutei are the products of twinning. The large pluteus, from an egg which failed to produce twins, serves as a control.

Figure 3. Second cleavage of *Dendraster* egg in Ca-free sea water, showing irregular positions of blastomeres. Eggs had been placed in Ca-free sea water at 30 minutes after fertilization and exposed for 60 minutes, during which time the first and second cleavages occurred.

Figure 4. Blastulae from eggs that had been exposed to Ca-free sea water from thirtieth to ninetieth minute after fertilization (*cf.* Fig. 3). This experiment paralleled that shown in Figure 1, used the same lot of fertilized eggs and was photographed at the same time as Figure 1. Rotating blastulae gave blurred photographs.

and half have passed into the insensitive stage following metaphase. The latter are blocked in the two-cell stage. Upon return to sea water after 30 minutes in mercaptoethanol, those that had divided in the mercaptoethanol gave rise to twin embryos. Those that were blocked before the first division gave single embryos. By the thirty-eighth minute after fertilization, all of the cells had passed the critical stage, divided in mercaptoethanol and gave rise to a large proportion of twin embryos. Around 45 minutes after fertilization, when most of the cells were well advanced in cleavage at the time the mercaptoethanol was introduced, the yield of twin embryos began to decrease rapidly. If mercaptoethanol was introduced 10 minutes later, the number of twins produced was small.

The critical time for twinning thus comes immediately after the critical time for mitotic blockage, as determined in the previous study (Mazia, 1958). The maximum yield of twins is obtained when the mercaptoethanol is introduced just at the time of the mitotic elongation of the cleavage furrows. The duration of the

TABLE I

Production of twin embryos from Dendraster eggs placed into 0.1 M mercaptoethanol at various times after fertilization

Time after fertilization when mercaptoethanol was introduced* (minutes)	Per cent cleavage in mercaptoethanol	Per cent twin blastulae**
35	50	50
38	90	85
41	95+	90
44	95+	45
47	95+	30
50	95+	8
53	95+	8

* Duration of mercaptoethanol treatment: 30 minutes.

** These percentages are relative to the total number of blastulae, and do not take into consideration individuals which degenerated before reaching the blastula stage. In the particular experiment from which Table I is taken, about 15 per cent of the eggs treated at 38, 41 and 44 minutes degenerated.

exposure to mercaptoethanol seems to have relatively little significance. What is important is that it acts during the brief effective period: the 5–10 minutes during which mitosis is completed and furrowing is going on.

Another kind of variation of "sensitivity" to mercaptoethanol with time should be mentioned, though it has not yet received adequate study. This is evidenced by the failure of some of the eggs to form normal blastulae, single or double. This was not recorded in Table I, where the fraction of twin blastulae is given relative to the total number of blastulae. To illustrate, in the experiment given in Table I, a yield of 90 per cent twins from eggs exposed at 41 minutes after fertilization was given. In the whole population, 15 per cent of the eggs failed to form normal blastulae, so that the yield of twins could also be given as 78 per cent—still a very high figure.

The best-studied methods of obtaining multiple embryos from echinoderm eggs involve modification of the ionic content of the environment, whether by removing

Ca or other ions or by varying the concentration of the sea water in the direction of hypotonicity or hypertonicity. In the present study, the effects of Ca-free sea water were compared with those of mercaptoethanol. A small volume of fertilized eggs (less than 0.5 ml.) was washed by centrifuging and re-suspending in 15 ml. of Ca-free sea water four times, beginning at 30 minutes after fertilization. The

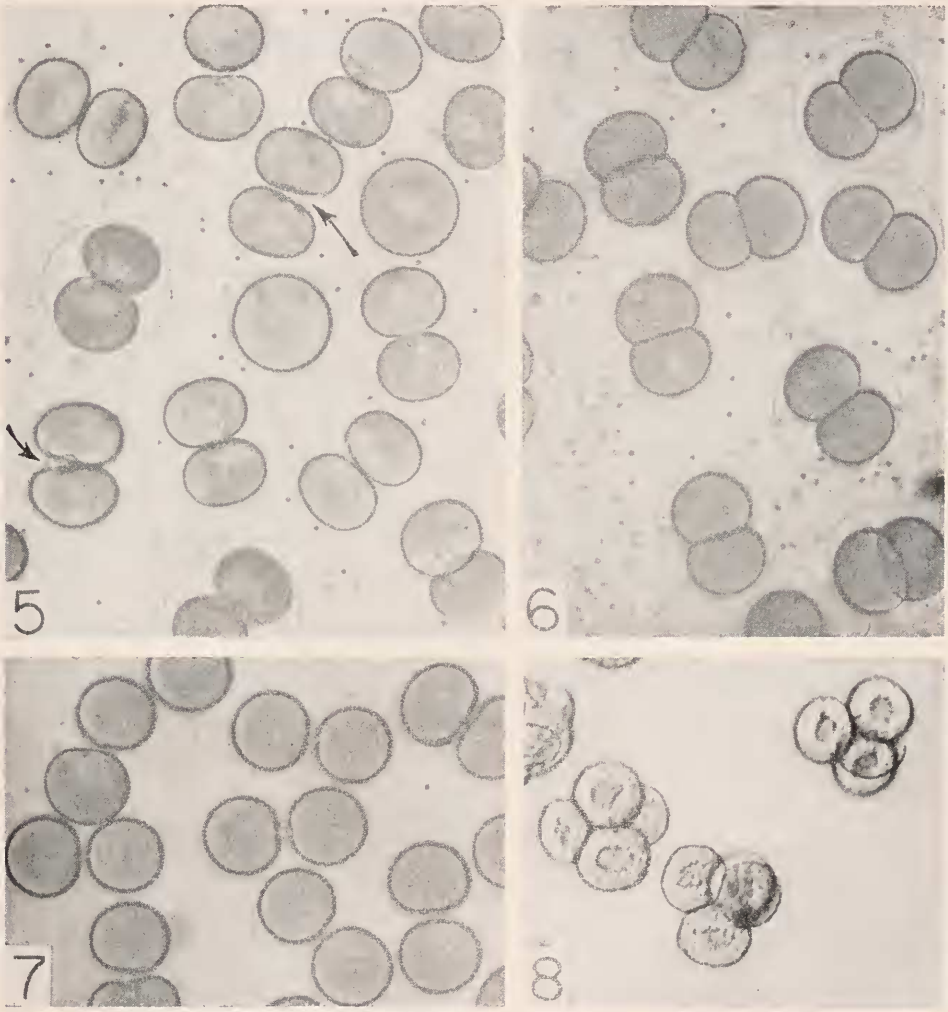

FIGURE 5. *Dendraster* eggs with fertilization membranes cleaving in 0.1 *M* mercaptoethanol. Blastomeres are not so firmly apposed as in control (Fig. 6), but appear to be connected by strands of clear material (arrows).

FIGURE 6. Control for Figure 5. Eggs have just completed cleavage in normal sea water.

FIGURE 7. *Dendraster* eggs without fertilization membranes just after cleavage in 0.1 *M* mercaptoethanol.

FIGURE 8. Quadruplets produced when mercaptoethanol treatment is applied at both the first and second divisions.

fertilization membranes were not removed. The eggs were permitted to go to the four-cell stage in Ca-free sea water before being returned to normal sea water. It was clear by this time that the Ca-deficiency was having its expected effect on blastomere adhesion. The first cleavage blastomeres were not flatly apposed, as was the case in the control, and the planes of the second cleavages did not coincide (Fig. 3). Nevertheless, long treatment with Ca-free sea water did not cause twinning (Fig. 4). Apparently, the embryo can organize itself to form a single blastula, following the treatment with Ca-free sea water, as long as the blastomeres are held together within the fertilization membrane. This corresponds with Harvey's (1940) experience with hypertonic sea water. It should be mentioned that the results in Figure 1 and Figure 4 were obtained with the same lot of eggs.

The mercaptoethanol does visibly affect the adhesion of the blastomeres. Figure 5 shows eggs that have cleaved in mercaptoethanol, the fertilization membrane being present. While they are compressed together, we do see rather more separation than in the controls (Fig. 6), and also see strands of glassy-appearing material between the blastomere surfaces in the furrow. If the fertilization membranes have been removed by protease, the cleavage in mercaptoethanol gives fully spherical blastomeres (Fig. 7), connected by tenuous strands, an appearance almost identical with that of membrane-free eggs that have cleaved in Ca-free sea water.

It would be predicted that if the mercaptoethanol was applied again at the time of the second cleavage, quadruplets would be produced. This was the case, as shown in Figure 8. The yield of quadruplets was never as high as that of twins. This would be expected from the fact that the eggs were not as synchronous in their second cleavage. In a desynchronized population a good many of the embryos will either be in a stage earlier than metaphase, at which they will merely be blocked, or at a stage later than the sensitive part of cleavage (Table I), in which case the mercaptoethanol will not be effective.

Finally, it should be mentioned that the effect of mercaptoethanol could not be duplicated with ethanol or with ethylene glycol, the analogs lacking the SH group. The latter may be considered the active center, and other SH compounds might have similar effects. The writer has found none that is comparably nontoxic and therefore usable at such high concentrations.

Discussion

Two questions demand discussion: (1) the chemical interpretation of the effect of mercaptoethanol in inducing twinning, and (2) the relation of the results to the earlier observations on twinning and on blastomere adhesion. The most reasonable interpretation of the chemistry of the observed effect is that the mercaptoethanol is affecting some interaction between the blastomeres that involves the formation of S—S bonds. This reagent is commonly used for reducing S—S bonds in proteins (Olcott, 1942). It has been seen that its analogs lacking the SH group are ineffective in inducing twinning. The results would be in accord with the hypothesis that the blastomere interaction depends on the formation of a gel serving as a cement between the blastomeres, and would fit equally well with a hypothesis calling for the establishment of fibrous connections between the blastomeres. The formation of protein gels by the establishment of intermolecular S—S bonds has been described by Huggins *et al.* (1951). The role of S—S bonds in

the formation of protein fibers is well known from studies on the keratins. Mercaptoethanol would be expected to block such intermolecular bonding by preventing the oxidation of the SH or by competing with protein SH.

The fact that the mercaptoethanol is effective only during a short period and is ineffective later would lead to the conclusion that we are dealing with the formation of the inter-blastomere links during the cleavage process itself. The mercaptoethanol is effective in preventing the formation of the links but not in splitting them once they are formed. This might mean that it acts by competition with protein SH in the formation of S—S bonds, not by reduction of S—S. It might also mean that the protein S—S becomes inaccessible to the reagent or that sufficient secondary bonds are formed, following the establishment of the intermolecular S—S links, to hold the structure together in the face of the reduction of the latter. In any event, the results imply that during cleavage, connections are formed between the blastomeres. The alternative explanation is that pre-existing factors tending to hold the blastomeres together (*e.g.* the hyaline layer as envisaged by Dan and Ono, 1952) undergo a change that renders them susceptible to mercaptothanol during the brief period of cleavage.

These results do not stand in contradiction to any of the previous observations regarding the induction of twinning by other means and especially by variations in the ionic environment. These have been interpreted, with some difference of opinion as to the details, as reflecting the significance of extracellular layers having the character of calcium proteinates (Moore, 1949; Hagström and Hagström, 1954). The physical properties of such layers are known to be affected by the ionic composition of the medium; obviously they will also depend on the protein-to-protein links that make their existence as stable masses possible. The contrast between the effects of Ca-free sea water and of mercaptoethanol in the present experiments is explicable on the assumption that the Ca-free sea water softens the layers but does not dissolve them quickly, while the mercaptoethanol actually solubilizes newly-appearing or pre-existing protein that would normally function in holding the blastomeres together. Thus the effect of Ca-free sea water might be reversible where the effect of mercaptoethanol was not.

The most striking feature of the mercaptoethanol effect is its complete irreversibility with respect to the division during which the reagent was applied, and its complete lack of effect on subsequent divisions. If it is applied at first cleavage, the blastomeres are "isolated" but subsequent divisions are normal, and the end result is fully normal twins in a large proportion of the population. If it is applied again at the second division, there is a fair yield of normal quadruplets. The results are not perfect: some incomplete twinning and occasional quadruplets are observed when the treatment takes place at the first division and some triplets are obtained when the treatment is given at the first and second divisions. On the whole, however, the results may be described as the effective and irreversible isolation of blastomeres by chemical means.

Summary

1. When *Dendraster* eggs are permitted to cleave in 0.1 *M* mercaptoethanol in sea water and then restored to normal sea water, a large proportion of the embryos develops as twins, producing normal twin plutei.

2. The effectiveness of the mercaptoethanol treatment is restricted to the short period during which the first cleavage furrows are forming.

3. If the treatment is repeated at the time of the second cleavage, quadruplets are produced.

4. Twins are not produced when the eggs cleave in Ca-free sea water.

5. The results are discussed in terms of the significance of the thiol groups of proteins for the interactions of blastomeres.

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